(FILE 'HOME' ENTERED AT 16:11:06 ON 18 JUN 2003)

FILE 'CAPLUS, MEDLINE, EMBASE, BIOSIS, LIFESCI, AGRICOLA' ENTERED AT 16:11:37 ON 18 JUN 2003

L1	201	S STICE S?/AU
L2	102	S L1 AND BOVINE
L3	6	S L2 AND PY=1996
L4	2	DUP REM L3 (4 DUPLICATES REMOVED)

STIC-ILL QP251.B5

From: Ton, Thaian
Sent: Wednesday, June 18, 2003 4:13 PM
To: STIC-ILL
Cc: Ton, Thaian
Subject: Article Request

I would like to request the following:

Eistetter et al. Devel. Growth and Differ. 31:275-282 (1989)

Biol Reprod. 1996 Jan;54(1):100-10.
Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer.
Stice SL, Strelchenko NS, Keefer CL, Matthews L.

Thank you very much.

Thái-An N. Ton
Patent Examiner
Art Unit 1632
Room: 12A16 CM1
Mailbox: 12E12 CM1
(703) 305-1019
09/809018

BIOLOGY OF REPRODUCTION 54, 100–110 (1996)

Pluripotent Bovine Embryonic Cell Lines Direct Embryonic Development Following Nuclear Transfer

Steven L. Stice,[1] Nick S. Strelchenko, Carol L. Keefer,[2] and Lee Matthews

American Breeder Service, DeForest, Wisconsin 53532

ABSTRACT

Nuclear transfer (NT) procedures were used to determine the in vivo developmental capacity of bovine embryonic cell lines derived from both morula- and blastocyst-stage embryos. These cell lines differed in morphology from trophoblast and endoderm-like cells. Regardless of initial donor embryo stage, cells in the resulting bovine embryonic cell lines had a small cytoplasmic/nuclear volume ratio and contained cytoplasmic vesicles. Developmental rates to blastocyst stage for NT embryos were improved when smaller cells (15 μm) rather than larger cells (18 μm or 21 μm) were used in the NT procedure and the recipient oocyte was activated after the cell fusion step. NT embryos produced from these embryonic cell lines, both morula- and blastocyst-derived, initiated pregnancies following transfer into recipient females. However, all of these pregnancies were lost prior to 60 days of gestation. These NT embryos were able to direct development through organogenesis, with one NT fetus reaching 55 days before death. When viable NT embryos were recovered during early gestation (38 days), an absence of cotyledons and a hemorrhagic response in the caruncles were observed. A chimera produced by aggregating an NT embryo with two 8-cell-stage blastomeres from in vitro-produced embryos developed through the 85th day of gestation. However, this conceptus was also deficient of cotyledons. DNA markers indicated that 50% of the chimera conceptus tissues were derived from the embryonic cell line. Blastocyst- and morula-derived embryonic cell line nuclei are pluripotent in that they can direct development through organogenesis, with subsequent pregnancy loss due, at least in part, to a deficiency in placentome development.

INTRODUCTION

The establishment of embryonic stem (ES) cells from embryos of domestic farm animals has been problematic. There have been numerous reports of embryonic cell lines derived from domestic farm animal species that exhibit in vitro pluripotent characteristics (cattle [1], sheep [2, 3], pigs [2–4]). These cell lines can maintain a normal karyotype through numerous passages and are able to differentiate and form embryoid bodies in vitro. However, evidence of in vivo developmental capacity is lacking for most of these lines. Recent phenotypical information suggests that chimeric pigs [5] and rats [6] have been produced, but the embryonic cell line contribution to the germ cells remains undetermined. One method of obtaining immediate germ-line contribution would be to utilize ES cells as donor nuclei in the nuclear transfer (NT) procedure. Reports of NT calves derived from inner cell mass (ICM) cells [7, 8] and cultured ICM cells [9] suggest that embryonic cell lines derived from ICM or blastomeres used in NT procedures might result in fetuses or offspring.

In the mouse, the particular ES cell line and embryo manipulation technique used influence the ability of ES cells to differentiate into fetal and placental tissues. Mouse ES cells showed limited potency when used as donor nuclei in NT procedures [10]. ES cell NT embryos directed development to the blastocyst stage but failed to develop further when transferred to recipient animals. Possible causes for the pregnancy losses in the mouse could not be elucidated. However, Nagy and coworkers [11] showed that mouse ES cells from one particular ES cell line formed live offspring when aggregated to a tetraploid embryo. In those experiments, the tetraploid cells gave rise to placental tissue while the ES cell contribution was almost exclusively fetal. Taken together, the minimal mouse ES cell contribution to the placental tissues in the chimera studies, and the limited development of NT embryos, suggest that ES cells may not be capable of initially differentiating into all conceptus cell types.

In the present study, we tested the potency of bovine embryonic cell lines using NT procedures. Developmental rates to blastocyst stage for NT embryos were improved through modification in NT procedures. However, the developmental potential of NT embryos beyond the blastocyst stage was limited to the period of organogenesis, with pregnancy loss due, at least in part, to a deficiency in placentome development.

MATERIALS AND METHODS

Production of Embryonic Cell Lines

Production of embryonic cell lines from blastocyst-stage embryos were described elsewhere [1] and modified in our laboratory. Briefly, primary cultures of embryonic fibroblasts were obtained from 14–16-day-old murine fetuses. After the head, liver, heart, and alimentary tract were aseptically removed, the embryos were minced and incubated for 30 min at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells were plated in tissue culture flasks and cul-

Accepted August 23, 1995.
Received March 6, 1995.

[1]Correspondence and current address: Steven L. Stice, Advanced Cell Technology Inc., 207 Paige Laboratory, University of Massachusetts, Amherst, MA 01003. FAX: (413) 545-6326; e-mail: stice@vasci.umass.edu

[2]Current address: Nexia Biotechnologies, Inc., 21,111 Lakeshore Rd., Box 183, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9.

tured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), penicillin (100 IU/ml), and streptomycin (50 µl/ml). Three to four days after passage, embryonic fibroblasts, in 35 × 10 Nunc culture dishes (Baxter Scientific, McGaw Park, IL), were treated with mitomycin C (10 µg/ml; Sigma Chemical Company, St. Louis, MO) in supplemented alpha-MEM for a minimum of 3 h. The fibroblasts were grown and maintained in a humidified atmosphere with 5% CO_2 in air at 37°C. Only culture plates that had a uniform monolayer of cells were used to culture the bovine embryonic cells.

Bovine embryos were nonsurgically collected or produced through use of in vitro procedures [12]. Blastomeres from morula, or the ICM of blastocysts, were partially disaggregated by a 10-min incubation in a 0.3% protease E (Sigma) in HEPES-buffered Hamster Embryo Culture Medium (HECM [13]) supplemented with 3 mg/ml BSA fraction V (Sigma). After removal of a majority of the trophoblast, a clump of cells containing the ICM was washed through 3 ml of HECM and plated directly onto the mitomycin C-blocked fibroblast cells. Disaggregated blastomeres were placed under the fibroblast feeder layer to initiate more contact with the feeder layer. A transfer pipette (50–100-µm diameter) was used to transfer the blastomeres between the feeder layer and culture plate. After 24 h in culture, the feeder layer usually reattached to the culture plate. The cell lines were maintained in a growth medium consisting of alpha-MEM supplemented with 10% FCS and 0.1 mM beta-mercaptoethanol (Sigma). Growth medium was exchanged every 2 to 3 days. Initial colonies were observed by the fourth day of culture and could be passaged between the seventh and tenth day. Only cells having the following three morphological features were isolated for passage: a small cytoplasmic/nuclear volume ratio, nuclei with multiple nucleoli, and cytoplasmic vesicles. Isolation of cells meeting these three criteria was accomplished by using a glass needle to cut out and separate the desired portions of the cell colony. These sections were lifted off the dish and mechanically disaggregated into clumps of cells by repeated pipetting through a small-bore pipette (50–100 µm). The disaggregated clumps were then passaged onto a new feeder layer. Only cell lines that had undergone fewer than 10 passages were used as nuclear donors in the NT procedure.

NT Procedures

Four NT experiments were performed to examine the time of activation and fusion, donor cell size, individual cell lines, and the ability of NT chimeras to develop in utero. NT procedures have been described previously [14, 15]. Briefly, after slaughterhouse oocytes were matured in vitro [12], the oocytes were stripped of cumulus cells and enucleated with a beveled micropipette at approximately 18 h post the beginning of maturation (hpm). Enucleation was confirmed through use of Hoechst 33342 (3 µg/ml; Sigma). Individual donor cells were then placed into the perivitelline space of the recipient oocyte. Blastomeres from morula were mechanically separated, whereas embryonic cell line cells were disaggregated by protease treatment (see above). Time of fusion varied from one experiment to another, ranging from 20 to 28 hpm. However, fusion pulse parameters were maintained at a constant 90 V for 14 µsec as described previously [15].

The procedure used to artificially activate oocytes has been described elsewhere [16]. The timing of activation was held constant (24 hpm). In brief, the activation procedure was as follows. NT embryos were exposed for 4 min to ionomycin (5 µM; CalBiochem, La Jolla, CA) in HECM supplemented with 1 mg/ml BSA and then were washed for 5 min in HECM supplemented with 30 mg/ml BSA. The NT embryos were then transferred into a microdrop of CR1aa culture medium [17] containing 0.2 µM 4-diethylaminopyridine (Sigma) and cultured at 39°C 5% CO_2 for 4–5 h. The embryos were washed in HECM and then placed in CR1aa medium in microdrop plates and cultured for 3–4 more days at 39°C and 5% CO_2. At all stages, oocytes and embryos were cultured in pre-equilibrated drops of medium (30–50 µl) under light-weight mineral oil.

After 4 days in culture, NT embryos were transferred into microdrops of CR1aa medium containing 10% FCS. At Day 7 (Day 0 = day of fusion and activation) the developmental rates to blastocyst stage was determined. On Day 7 or 8, a limited number of blastocysts from various groups were transferred into recipient females. The stage of estrous cycle of the recipient was matched with the stage of the embryo.

Production of Chimeras

To produce aggregate chimeras, two 8-cell-stage blastomeres were added to an 8-cell-stage NT embryo. NT embryos have reached the 8-cell stage by Day 2.5 of culture. At that time, 8-cell-stage in vitro-produced embryos (Day 2.5) were mechanically disaggregated through use of a transfer pipette, and two of the blastomeres were transferred through the slit in the zona of the NT embryo. These transferred blastomeres were placed in direct contact with the blastomeres of the NT embryo. The aggregate embryos were placed back into CR1aa supplemented with 3 mg/ml BSA and on the third or fourth day transferred into CR1aa supplemented with 10% FCS. Those embryos in which the combined cells had formed a blastocyst were transferred into recipient females.

Pregnancy Detection and Ultrasound Observations

Recipient females were monitored daily for return to estrus. Females not returning to estrus between 25 and 30 days of gestation were examined by ultrasound (Aloka 500, 7.5 MHz transducer; Corometrics Medical Systems, Wallingford, CT). The presence of a fetal heartbeat was the criterion for

considering a recipient to be pregnant. Those animals diagnosed as pregnant were monitored every week for the presence or absence of a fetal heartbeat. The time of pregnancy loss was arbitrarily set at midweek between the time a heartbeat was present and the time it was absent. Pregnancies were monitored weekly until Day 90 of gestation.

DNA Markers

DNA was isolated from the tissues of presumptive NT and chimeric fetuses (heart, muscle, skin, and placenta) and calves (blood) to determine parentage. Polymerase chain reaction amplification of cytosine/adenine repeat microsatellite markers (MGTG 4-B, TLGA 73, TLGA 57, TLGA 53, TLGA 263, TLGA 48, TLGA 122, TLGA 126, TLGA 227, TLGA 261, TLGA 325, and TLGA 245; Linkage Genetics, Salt Lake City, UT) was performed, and the amplification products were analyzed at Linkage Genetics and Grace Washington Research Group (Columbia, MD). Conceptus and calf DNA marker results were compared with the DNA marker profiles of the bulls used to produce embryos for both the established embryonic cell lines and the aggregate blastomeres. In addition, DNA marker profiles were run on the embryonic cell lines themselves and compared with those of the conceptuses and calves. In order to quantify the embryonic cell line contribution in the chimera experiment, DNA mixes composed of known ratios of DNA were used as standards for comparison with the putative chimeric tissues results.

Sex Determination

Sexing of embryonic cell lines was accomplished by amplification of Y chromosome-specific DNA sequences using an assay kit produced by AB Technology (Pullman, WA). Three to six cells were removed from the embryonic cell lines and from the mouse feeder layer. These cells were washed through serum-free PBS and placed into microcapillary tubes containing 10 μl sterile, deionized water. The samples were frozen-thawed to lyse the cells, and a reagent mixture containing the primers, nucleotide triphosphates, buffer solution, and *Taq* polymerase (Complete-Reaction-Mix, AB Technology) was added. The primers in the reaction mix were for a bovine-ovine-caprine-specific Y chromosome repeat sequence and for a repeated autosomal sequence (control). Capillary tubes containing the samples, the control without DNA, and male and female controls were placed into a Corbett Capillary DNA Thermal Cycler FTS-1S (AB Technology). Male and nonspecific (autosomal control) DNA bands were separated and visualized by gel electrophoresis in an ethidium bromide-3% agarose gel. No bands were visible for the mouse feeder layer cells, demonstrating that the mouse feeder layer DNA did not interfere with this bovine-specific assay.

Histology

Conceptuses were recovered from five recipient animals by hysterectomy via a flank incision. Histological preparations were made of recovered fetuses and placentomes. Tissue was placed in 10% buffered formalin, dehydrated in increasing concentrations of ethyl alcohol, cleared in chloroform, and infiltrated with paraffin. Tissues were sectioned and stained with hematoxylin and eosin. Slide-mounted sections were then viewed under a microscope, and comparisons between normal conceptuses and NT conceptuses were made by a veterinary pathologist.

Statistical Analysis

Statistical computations were made through use of chi-square analysis for all data (Instat; GraphPad Software, San Diego, CA).

RESULTS

Establishment of Embryonic Cell Lines

Embryonic cell lines were established from in vivo- and in vitro-produced blastocyst- and morula-stage embryos (Table 1). Cell colony formation was observed approximately 3 to 7 days after initiation of culture (Fig. 1a). An embryonic cell line was considered established when a homogenous population of cells could be maintained for several generations. Embryonic cell lines were established from all types of embryos tested. Each cell line maintained a low cytoplasmic to nuclear ratio, prominent nucleoli in the nucleus, and lipid vesicles in the cytoplasm. Some cell lines were allowed to proliferate, maintaining proper morphology for over 50 passages (over 12 mo of in vitro culture). Furthermore, cell lines from each of the four types of embryos formed embryoid bodies when colonies became overcrowded in vitro (Fig. 1d). These structures were considered embryoid bodies because of their resemblance to blastocyst-stage embryos and the differentiation of cells within the structure (muscle, red blood cells; S.L. Stice, personal observations). In general, there were no morphologically distinguishable differences among embryonic cell lines derived from various stages (blastocyst or morula) and types (in vitro or in vivo derived) of donor embryos (Table 1).

Besides forming embryoid bodies, the embryonic cell lines also formed differentiated cell types in vitro. Large, loosely attached, highly refractory, and irregularly shaped endoderm-like cells (not shown) were present as were trophectoderm-like cells (Fig. 1b). Trophectoderm-like cells were often present in the primary culture and later removed and discarded. These cells had distinguishing features that included a larger cytoplasmic/nuclear volume ratio than the embryonic cell lines and had numerous cytoplasmic vacuoles. When dissociated, trophectoderm-like cells were not round in shape and were obviously larger in diameter than

TABLE 1. Derivation of embryonic cell lines derived from different stages and sources of embryos.

Embryo stage	Source of embryo	No. of embryos placed in culture	No. of established cell lines*	% Established	Formed embryoid bodies
Morula	In vitro	12	8	67%	Yes
Morula	In vivo	11	6	55%	Yes
Blastocyst	In vitro	15	7	47%	Yes
Blastocyst	In vivo	27	15	55%	Yes

*Numbers of established embryonic cell lines were not different among embryo stages and sources.

the embryonic cell line cells (Fig. 1c). Disaggregated trophoblast cells obtained directly from a blastocyst-stage embryo had similar trophectoderm-like characteristics.

NT

Experiment I: Time of activation. Previously it was reported that temporal relationship between activation and fusion affected the developmental rate of bovine 32-cell-stage blastomere-derived NT embryos [15, 18, 19]. Therefore, developmental rates were determined for NT embryos in which the donor cells (blastomeres or embryonic cell line cells) were fused with the enucleated oocyte either before, simultaneously with, or after oocyte activation (Table 2). The temporal relation between activation and fusion did affect developmental rates, but embryonic cell line nuclei

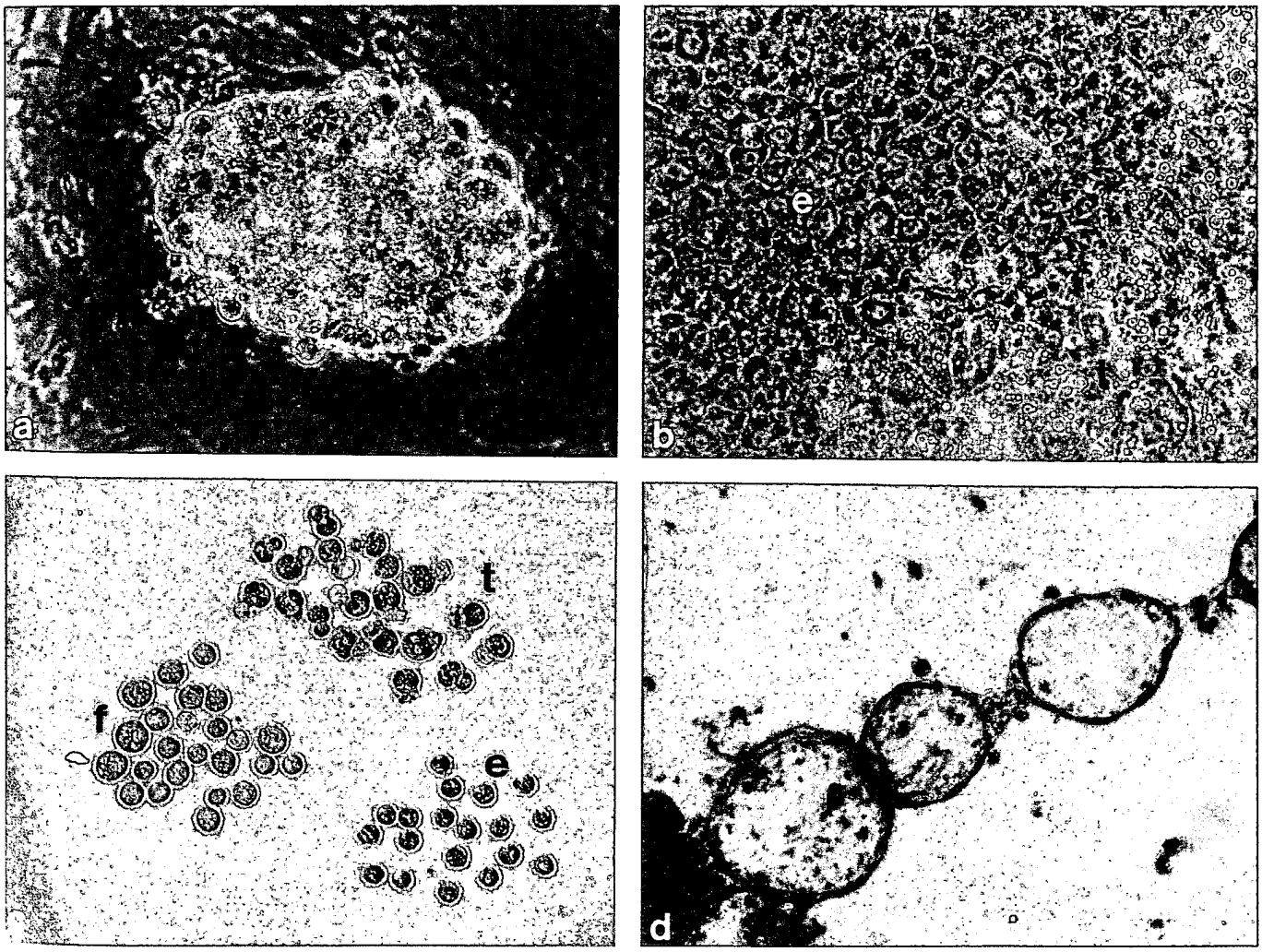


FIG. 1. a) A typical cell colony from morula- and blastocyst-stage embryos 4–7 days after being seeded on top of a feeder layer. b) An embryonic cell line (e) colony exhibiting a high nuclear-to-cytoplasmic ratio and some vesicles in the cytoplasm. An outgrowth of trophoblast cells (t) developing around the perimeter of a colony. c) Disaggregated cells, mouse embryonic fibroblast cells (f), trophoblast cells (t), and embryonic cell line cells (e). d) Simple embryoid body or vesicle formation after overcrowding of cell colonies in culture. a-c, ×186, original ×200; d, ×93, original ×100.

TABLE 2. The effect of time of activation in relation to time of fusion on development to blastocyst and establishment of pregnancies in NT embryos derived from either blastomere or embryonic cell line nuclei.

Type of donor nuclei	Time of fusion*	No. of donor cell lines or embryos	No. NTs produced	No. NTs to blastocysts (%)	No. NTs transferred to recipients	No. pregnant (%)[c]		
						> 30 Days	> 40 Days	> 50 Days
32-cell blastomere	20 h	4	74	0[a] (0)	—	—	—	—
Embryonic cell line	20 h	3	1045	103[b] (10)	45	6 (13)	1 (2)	0
32-cell stage blastomere	24 h	4	105	7[b] (7)	3	0 (0)	0	0
Embryonic cell line	24 h	3	1075	29[a] (3)	19	1 (5)	0	0
32-cell stage blastomere	28 h	15	475	62[b] (13)	10	3 (30)	3 (30)	2 (20)
Embryonic cell line	28 h	7	2195	47[a] (2)	41	3 (7)	1 (2)	0

*Time of fusion varied (20, 24 or 28 hpm), but time of activation remained constant (24 hpm).
[a,b]Number developing to the blastocyst stage without a common superscript differed ($p < 0.05$).
[c]Too few of the NT embryos were transferred to determine differences in pregnancy rates.

responded differently than did the blastomere nuclei. As expected, developmental rates were highest for blastomere-derived NT embryos when the blastomeres were fused (28 hpm) with previously activated oocytes (13%; $p < 0.05$). The embryonic cell line NT embryos' highest developmental rates to blastocyst stage (10%) were obtained when the NT embryo was activated 4 h after the cell fusion procedure (20 hpm). Although the fusion pulse might have induced partial activation of NT embryos, complete activation was accomplished only after the activation stimulus was given 4 h later. NT embryos not receiving the activation stimulus after fusion underwent premature chromatic condensation and did not cleave. These nonactivated NT embryos were also used to determine fusion rate. Fusion rates did not differ among experimental groups in this study (data not presented). In this initial experiment, a limited number of NT blastocysts were transferred to recipient females. Only the blastomere NT embryos developed past 50 days of pregnancy and eventually to full term (Table 2). Although all of the embryonic cell line NT pregnancies eventually aborted, the pregnancies had clearly been established as determined by ultrasound.

Experiment II: Donor cell size. The relative size of mouse ES cells used to produce chimeric embryos has been shown to be correlated with resulting percentage of germline chimeras [20]. Smaller-diameter mouse ES cells resulted in a higher percentage of germ-line chimeras. Therefore, the effect of cell diameter on developmental rates to the blastocyst stage after NT was monitored. Following disaggregation, individual cells were categorized into three different groups: large, medium, and small cells with approximate diameters of 21 μm, 18 μm, and 15 μm, respectively. Cells from each group were used in the NT procedure. The fusion pulse (20 hpm) was applied 4 h prior to the activation stimulus. Developmental rates were affected by the size of the donor cell (Table 3), with small cells having significantly higher developmental rates to the blastocyst stage than medium or large cells ($p < 0.005$). Only 19 NT embryos derived from these cell groups were transferred to recipient animals, and those that were transferred failed to maintain the pregnancy beyond 39 days of gestation (Table 3).

Experiment III: Individual cell lines. Embryonic cell lines derived from various sources of embryos were used as donor nuclei in the NT procedures. The NT parameters that yielded the highest developmental rates to the blastocyst stage in experiments I and II (i.e., fusion prior to activation and small cells) were used in this experiment. Developmental rates to the blastocyst stage as well as pregnancy rates were monitored (Fig. 2a). Of the sexed cell lines, the male line had a lower developmental rate to the blastocyst stage than did the female lines ($p < 0.001$). Conversely, NT blastocysts derived from the male line (0761) had a higher proportion of established pregnancies developing past 40 days (6 of 39, 15%) than did those from the female lines (pooled results, 0734 and 0713; 9 of 177, 5%; $p < 0.05$). Two conceptuses reached greater than 50 days of gestation before the heartbeats were no longer detected during ultrasound examination.

Five conceptuses ranging from 35 to 55 days of gestation were recovered after their heartbeat was no longer detected by ultrasound. A 55-day fetus showed no gross abnormalities when recovered (Fig. 2b). Similarly, the other four conceptuses exhibited no overt abnormalities. Two of the five fetuses were prepared for histological analysis and DNA marker analysis. DNA marker identification confirmed that these fetuses were derived from the embryonic cell line

TABLE 3. The development of NT using donor nuclei from embryonic cell line cells of various sizes derived from blastocyst-stage embryos with activation occurring 4 h after fusion.

Embryonic cell line cell diameter	No. NTs produced	No. NTs to blastocyst (%)	No. NTs transferred to recipients	No. pregnant	
				> 30 Days	> 40 Days
15 μm	592	117[a] (20)	11	0	—
18 μm	703	88[b] (13)	4	1	0
21 μm	643	77[b] (10)	4	0	—

[a,b]Numbers developing to the blastocyst stage without a common superscript differ ($p < .0005$).

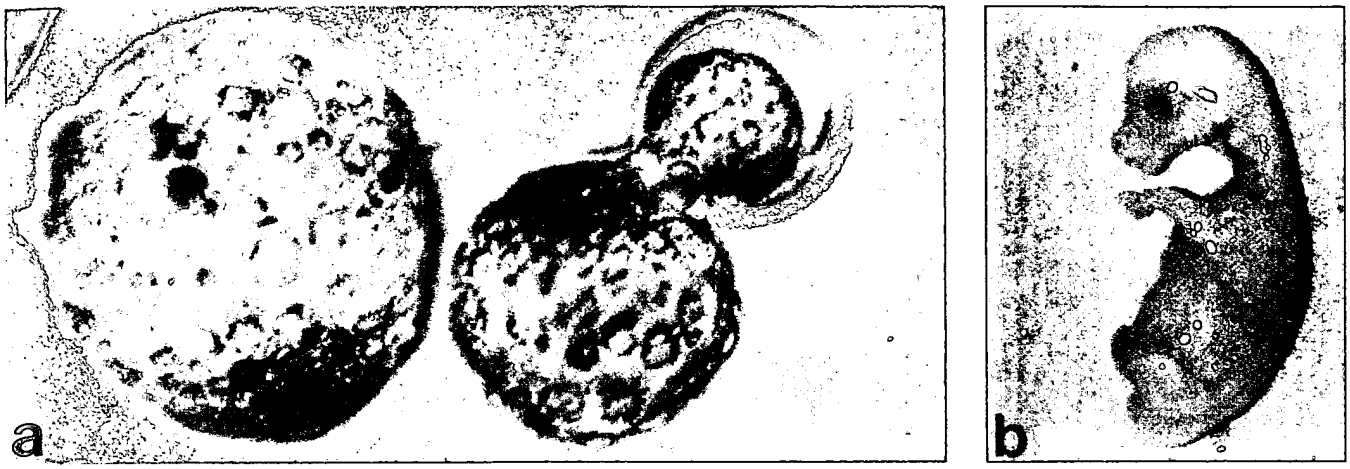


FIG. 2. a) Day 8 blastocyst-stage embryos derived from embryonic cell lines (×200). b) A Day 55 fetus derived from an embryonic cell line NT embryo (shown actual size).

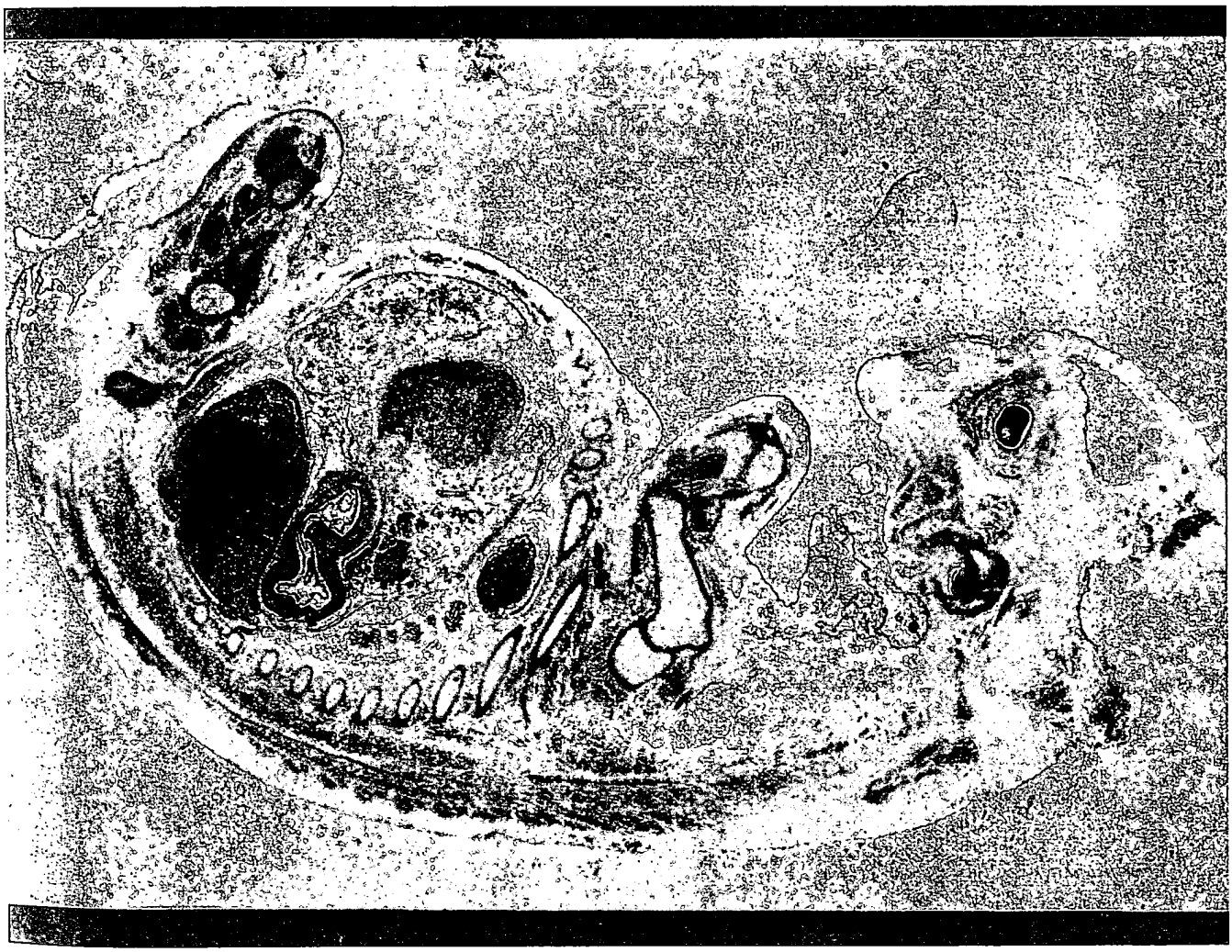


FIG. 3. A sagittal section of a Day 40 fetus derived from an embryonic cell line NT embryo (×4).

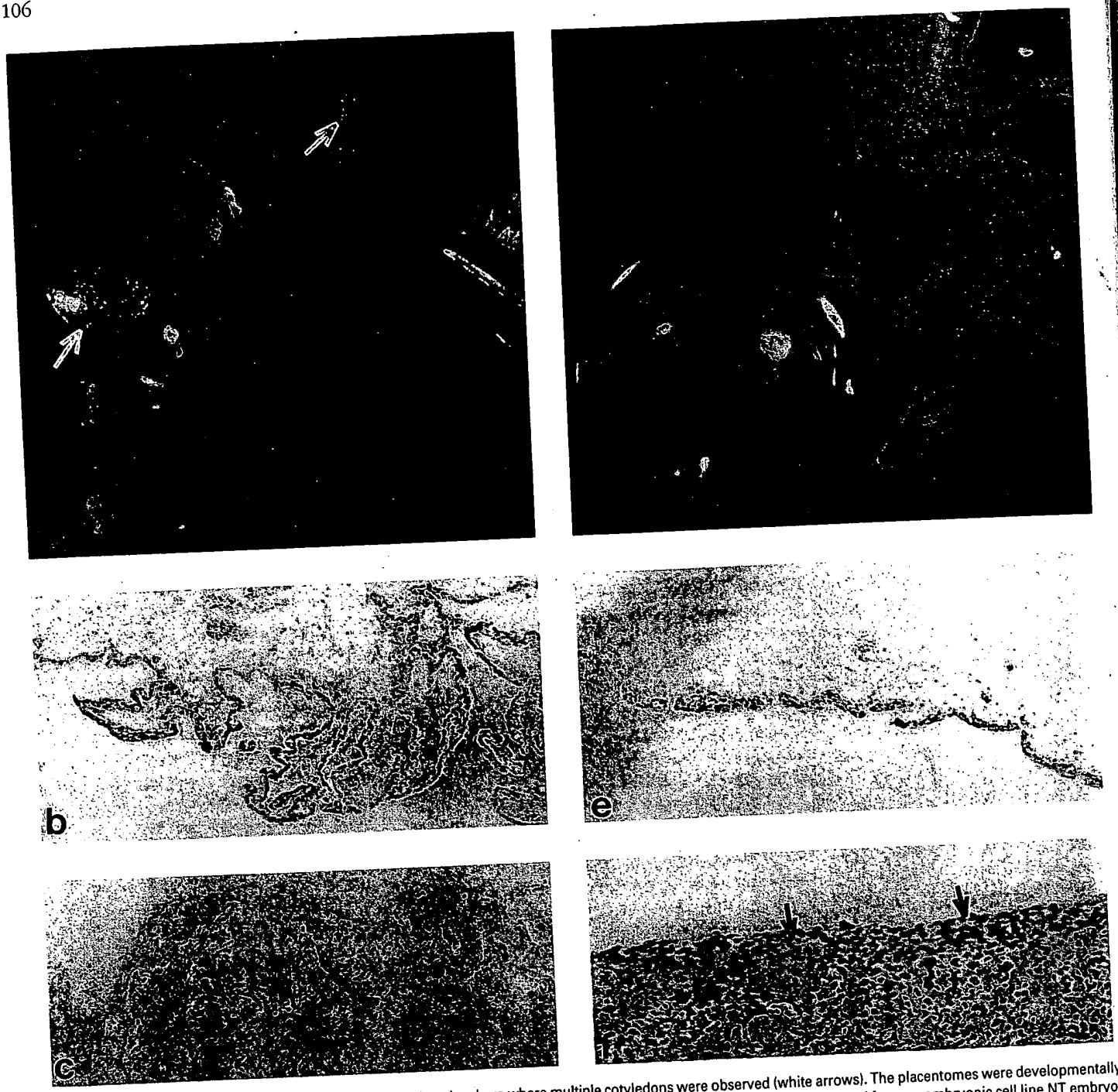
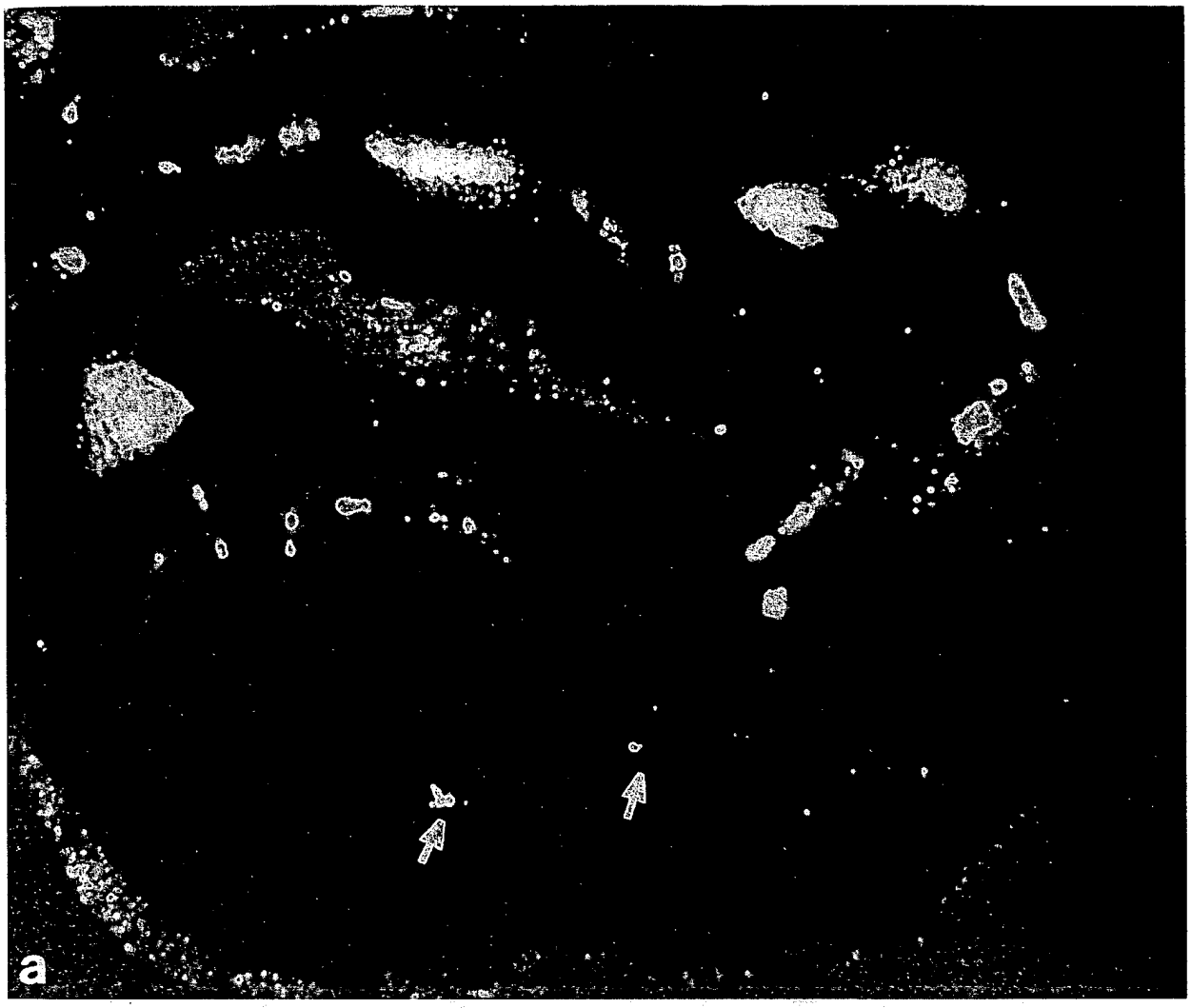


FIG. 4. a-c) Day 38 conceptus derived from an in vitro-produced embryo where multiple cotyledons were observed (white arrows). The placentomes were developmentally normal with cotyledons (b) and caruncles (c) forming villi and crypts, respectively, at this time point. d-f) Day 38 conceptus derived from an embryonic cell line NT embryo. Note the absence of cotyledonary structures (d), an absence of villi in the placental tissue (e), and caruncles (f) void of crypts. The caruncular tissue also had large areas containing erythrocytes near the surface epithelium (black arrows). a and d, shown actual size; b,c,e,f, ×100.

0734. Sagittal sections from these fetuses were not informative as to the cause of death in these pregnancies (Fig. 3). However, gross examination of the placental tissue of the NT conceptuses showed that cotyledonary tissue was obviously absent (Fig. 4, d and e). Binucleate cells were present in the placental membranes of the NT pregnancies. However, whether these cells were physiologically normal

FIG. 5. a) An 85-day chimeric conceptus (shown at 95% of actual size) derived from an embryonic cell line NT embryo (50% contribution) aggregated with a fertilized embryo (50% contribution). This placenta was largely deficient of cotyledons; however, a few rudimentary structures were observed (arrows). b) The fetus from the same conceptus appeared normal (×1.4).


a


b

TABLE 4. The development of NT using donor nuclei from embryonic cell lines derived from morula- or blastocyst-stage embryos using 15-μm donor cell and activation 4 h after fusion.

Embryonic cell line ID	Stage	Source	Sex	No. NTs produced	No. blastocysts (%)	No. blastocysts transferred to recipients	No. pregnant (%)		
							> 30 Days	> 40 Days	> 50 Days
0734	Blastocyst	In vivo	F	1047	246[a] (23)	164	27 (16)	9[d] (5)	1 (1)
0761	Blastocyst	In vivo	M	498	58[b] (12)	39	8 (21)	6[e] (15)	1 (3)
0713	Blastocyst	In vitro	F	863	314[c] (36)	13	4 (31)	0[d]	0
0870	Morula	In vivo	—	84	34[c] (40)	15	2 (13)	0	0
0677	Morula	In vitro	—	704	250[c] (36)	20	6 (30)	1 (5)	0

*Pregnancy rates did not differ at 30 days.
[a,b,c]Numbers developing to the blastocyst stage without a common superscript differ ($p < 0.001$).
[d,e]Numbers pregnant past 40 days without a common superscript did differ among the blastocyst-derived cell lines when results within the same sex were pooled ($p < 0.05$).

remains unknown. Four conceptuses derived from in vitro-produced embryos taken at similar times (30–55 days of gestation) possessed cotyledonary tissue. Furthermore, the caruncular tissue from the NT pregnancies did not develop crypts and had a hemorrhagic response at the surface of the caruncles (Fig. 4f). In all cases, NT embryo placentation was abnormal for that stage of gestation.

Experiment IV: Aggregation chimeras. In an attempt to rescue the NT pregnancies, aggregate chimeras were produced by combining the 8-cell-stage blastomeres from NT embryos with two blastomeres from in vitro-produced embryos of a similar stage (Fig. 1b). Aggregate embryos (n = 53) were transferred to recipient females. Initially, eight pregnancies were established past 60 days of gestation. Three pregnancies were lost between 60 and 90 days of gestation, and five pregnancies were carried to term. One of the pregnancies that aborted at 85 days was recovered (Fig. 5). DNA microsatellite markers were used to establish parental origin and percentage of embryonic cell line contribution to the chimeras. Tissue from the placenta, skin, muscle, and heart showed approximately a 50/50 contribution from the two embryos used to make the aggregate chimeras. Again, no abnormalities were observed in the fetus, but abnormally fewer and smaller cotyledons were present on the placenta than expected for that stage of gestation (Fig. 5). There was no detection of embryonic cell line contribution to the blood and ear punch samples obtained from the offspring. Sensitivity of the DNA marker assay should allow the detection of an embryonic cell line contribution of 10% or greater in any tissue.

DISCUSSION

Established bovine embryonic cell lines directed embryonic development through organogenesis when used as donor nuclei in NT procedures. In order to make conclusive genetic identification of fetuses and offspring using DNA markers, the marker genotype of either the maternal and paternal source or, as in the present study, the embryonic cells themselves must be analyzed. DNA marker results confirmed that embryonic cell lines did contribute to NT fetuses and multiple tissue types in an 85-day NT chimeric fetus. Marker analysis of offspring indicated that there was little or no genetic contribution from the embryonic cell lines to blood or ear tissues samples.

The exact cause(s) of pregnancy loss for both NT embryos and chimeras is unknown. However, placental developmental failure should be considered a contributing factor. Normally, cotyledon formation occurs by Days 25–30 of gestation in cattle [21, 22]. It has been reported that until approximately Day 40, it is possible for the bovine conceptus to survive on "uterine milk" [21]. However, after this time point, fetal-maternal exchange of nutrients and gases must occur through functioning placentomes in order to maintain the pregnancy. The timing of pregnancy losses in the present study coincides with timing of placentome formation in normal conceptuses. The hemorrhaging in the caruncles of NT pregnancies indicated that there was a maternal response to the presence of the conceptus but that proper placentome formation did not ensue. There was also a marked reduction in the number and size of cotyledons in the chimeric pregnancy, indicating that the contribution of the in vitro-produced embryo could prolong the pregnancy but not totally rescue it. Future chimera experiments in which the placental contribution from the NT embryo is minimized might extend these pregnancies past 85 days of gestation.

The timing of fusion in relation to activation and the size of the donor cell had a significant effect on NT embryo development rates to the blastocyst stage. Fusion (20 hpm) at 4 h prior to activation (24 hpm) was most beneficial for embryonic cell line NT embryos, whereas for blastomere NT embryos, the developmental rates were higher when fusion (28 hpm) occurred at least 4 h after activation (24 hpm). In previous studies [15, 18, 19], the fusion pulse has been used to activate aged oocytes (> 30 hpm). Thus, it is possible that the fusion pulse might have also induced oocyte activation in the present study. However, NT embryos were not activated when given a fusion pulse alone (20 hpm), suggesting that fusion pulse-induced activation was negligible in this study. The data for blastomere NT embryos were in agreement with results from previous bovine studies in which developmental rates were improved when the recipient oocytes

were activated prior to blastomere fusion [15, 18, 19]. The cell cycle stage of the donor blastomere has also been shown to affect bovine NT embryo developmental rates. The optimal developmental rates occurred when 32-cell-stage bovine blastomeres (10–15 h after M phase) were fused with a previously activated oocyte [23]. The cell cycle of blastomeres differs greatly from that of the embryonic cell lines. Approximately 80% of cleavage-stage bovine blastomeres are in the S phase [18], whereas 40–60% of embryonic cell line cells are in G1 phase at any given time (S.L. Stice and N.S. Strelchenko, unpublished results from flow cytometry analysis). Dissimilarities in the cell cycle characteristics might account for differences in developmental rates observed between these two types of donor nuclei. In the rabbit, a blastomere in G1 phase had a higher developmental rate to the blastocyst stage when placed in a nonactivated M-phase oocyte [24]. As most of the embryonic cell line nuclei in the present study resided in G1 phase, resulting NT embryos had a higher development capacity when these nuclei were fused with a nonactivated oocyte.

In a study in the mouse, the percentage of offspring that were chimeric increased when smaller (7–9 μm) rather than larger (9–18 μm) ES cells were injected into the blastocoele cavity of the host blastocysts [21]. Similarly to mouse ES cell chimera experiments, the present studies showed that individual bovine embryonic cells with a smaller cell diameter (15 μm) had a higher developmental capacity than larger cells (18 or 21 μm). Perhaps smaller cells have divided more recently and therefore were earlier in the cell cycle (G1) than the larger cells. Alternatively, the larger cells might have been polyploid and thus less likely to develop to the blastocyst stage when used as donor nuclei in the NT procedure. A proportion of cells within bovine embryonic cell lines have been shown to possess an abnormal karyotype [25]. In any case, the status of the cell cycle and the cell's ploidy are important factors to consider when one attempts to improve the efficiency of NT procedures.

Similarities in the pluripotent properties between the bovine embryonic cell lines in the present study and mouse ES cells in other studies were observed. NT embryos derived from mouse ES cells [10] and NT embryos derived from the bovine embryonic cell line developed to the blastocyst stage, but in both cases the resulting pregnancies were either reabsorbed (mouse) or lost early in gestation (bovine). Furthermore, tetraploid cells aggregated with mouse ES cells resulted in conceptuses with placental tissue almost exclusively derived from the tetraploid cells [11], indicating a failure of the ES cells to make a major contribution to the placenta. Likewise, the bovine embryonic cells were not able to direct normal placentome formation. While chimerism prolonged development of the conceptus to 85 days, the in vitro-produced embryo contribution to the placenta was only 50%. Perhaps a higher contribution from these cells would extend pregnancies further. Bovine tetraploid embryos did not develop beyond the 8-cell-stage in our laboratory (data not presented), thus prohibiting the use of tetraploid embryos as helper cells in placentation for bovine NT embryos.

From the male and female bovine embryonic cell lines studied (Table 4), sex- or cell line-dependent differences appear to exist. The male line (0761) derived from a blastocyst-stage embryo had a higher proportion of resulting NT embryos developing past 40 days of gestation than did similar lines derived from female embryos (0734 and 0713). Male mouse ES cell lines are more easily obtained, and they maintain a more stable karyotype than female lines [26]. Bovine embryonic cell lines do undergo karyotypic changes over time, and female lines have an increase in X-inactivation over 15 passages [25]. Subtle differences in genomic stability might explain developmental differences observed among embryonic cell lines used in this study. Additional experiments are needed to determine whether or not these differences are related to the sex of a particular cell line.

Differences in pluripotent properties between morula- and blastocyst-stage-derived embryonic cell lines were not observed in the current study. Pluripotent mouse ES cells have been produced from mouse morula-stage embryos but have not been tested for germ-line contribution [27]. Mink embryonic cell lines derived from morula-stage embryos give rise to more diverse differentiated tissue types than cell lines derived from early blastocysts [28]. However, the techniques used to determine the potency of the cell lines differed greatly among these studies. Species-specific differences may also explain these observed differences.

Previous bovine NT investigations using blastomeres as donor nuclei also showed a high incidence of pregnancy loss and placental abnormalities. The greatest number of established pregnancies were lost between Days 30 and 90 of gestation [29]. In addition, multiple generational cloning leads to a higher incidence of pregnancy losses ([30]; K. Bondioli, personal communications). In fact, all pregnancies produced from greater than three generations of recloning have aborted prior to 60 days of gestation (S.L. Stice, unpublished results). A higher incidence of hydrops or hydrallantois, an abnormal accumulation of fluid in the allantoic cavity, has been observed with blastomere NT pregnancies [29]. Therefore, blastomere NT embryos also appear to have placental development deficiencies, albeit not to the extent observed with embryonic cell line NT embryos.

In summary, bovine embryonic cell lines derived from either morula- or blastocyst-stage embryos exhibit in vivo pluripotential properties. Pluripotency was demonstrated through NT procedures resulting in NT embryos that developed through early organogenesis. Developmental capacity of NT embryos could be extended out to 85 days when they were aggregated with cleavage-stage in vitro-derived embryos. Pregnancies should be extended even further when placental deficiencies observed in both the NT and chimera are reduced or eliminated.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Jeff Betthauser, Mr. Paul Golueke, Ms. Gail Jurgella, Ms. Ann Marie Paprocki, and Mr. Brett Scott for their technical assistance and Ms. Rita Wells for help in preparing this manuscript. In addition, we appreciate the technical assistance in DNA marker analysis from Mr. Victor David and Dr. Jim Jackson of W.R. Grace.

REFERENCES

1. Saito S, Strelchenko N, Niemann H. Bovine embryonic stem cell-like cell lines cultured over several passages. Roux's Arch Dev Biol 1992; 201:134–141.
2. Notarianni E, Laurie S, Moor RM, Evans MJ. Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. J Reprod Fertil Suppl 1990; 41:51–56.
3. Piedrahita JA, Anderson GB, BonDurant RH. On the isolation of embryonic stem cells: comparative behavior of murine, porcine and ovine embryos. Theriogenology 1990; 34:879–901.
4. Strojek RM, Reed MA, Hoover JL, Wagner TE. A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts. Theriogenology 1990; 33:901–913.
5. Wheeler MB. Development and validation of swine embryonic stem cells: a review. Reprod Fertil Dev 1994 6:563–568.
6. Iannaccone PM, Taborn GU, Garton RL, Caplice MD, Brenin DR. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. Dev Biol 1994; 163:288–292.
7. Keefer CL, Stice SL, Matthews DL. Bovine inner cell mass cells as donor nuclei in the production of nuclear transfer embryos and calves. Biol Reprod 1994; 50:935–939.
8. Collas P, Barnes FL. Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei. Mol Reprod Dev 1994; 38:264–267.
9. Sims M, First NL. Production of calves by nuclear transfer of nuclei from cultured inner cell mass cells. Proc Natl Acad Sci USA 1993; 91:6143–6147.
10. Tsunoda Y, Kato Y. Nuclear transplantation of embryonic stem cells in mice. J Reprod Fertil 1993; 98:537–540.
11. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci USA 1993; 90:8424–8428.
12. Keefer CL, Stice, SL, Dobrinsky J. Effect of FSH and LH during bovine in vitro maturation on development following in vitro fertilization and nuclear transfer. Mol Reprod Dev 1993; 36:469–474.
13. Seshagiri PB, Bavister BD. Phosphate is required for inhibition by glucose of development of hamster 8-cell embryos in vitro. Biol Reprod 1989; 40:607–614.
14. Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone W, First NL. Nuclear transfer in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 1987; 37:859–866.
15. Stice SL, Keefer CL, Matthews L. Bovine nuclear transfer embryos: oocyte activation prior to blastomere fusion. Mol Reprod Dev 1994; 38:61–68.
16. Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Shutzkus V, First NL. Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev Biol 1995; 729–739.
17. Rosenkrans CF, First NL. Culture of bovine zygotes to the blastocyst stage: effects of amino acids and vitamins. Theriogenology 1991; 35:Abstract 266.
18. Barnes FL, Collas P, Powell R, King WA, Westhusin M, Shepherd D. Influence of recipient oocyte cell cycle stage on DNA synthesis nuclear envelope breakdown, chromosome constitution, and development in nuclear transfer bovine embryos. Mol Reprod Dev 1993; 36:33–41.
19. Kono T, Sotomaru Y, Aono F, Takahasi T, Ogiwara I, Sekizawa F, Arai T, Nakahara T. Effect of ooplast activation on the development of oocytes following nucleus transfer in cattle. Theriogenology 1994; 41:1463–1471.
20. Brown DG, Willington I, Findley I, Muggleton-Harris AL. Criteria that optimize the potential of murine embryonic stem cells for in vitro and in vivo developmental studies. In Vitro Cell Dev Biol 1992; 28A:773–778.
21. Melton AA, Berry RO, Butler OD. The interval between the time of ovulation and attachment of the bovine embryo. J Anim Sci 1951; 10:993–1005.
22. King GJ, Atkinson BA, Robertson HA. Development of the placentome from days 20 to 29 of gestation. J Reprod Fertil 1980; 59:95–100.
23. Stice SL, Keefer CL, Maki-Laurila M, Matthews L. Donor blastomere cell cycle stage affects developmental competence of bovine nuclear transfer embryos. Theriogenology 1993; 39:Abstract 318.
24. Collas P, Balise JJ, Robl JM. Influence of cell cycle stage of donor nucleus on development of nuclear transplant rabbit embryos. Biol Reprod 1992; 46:492–500.
25. Strelchenko N, Miltipova M, Stice SL. Further characterization of bovine pluripotent stem cells. Theriogenology 1995; 43:Abstract 327.
26. Robertson EJ, Kaufman MH, Bradley A, Evans MJ. Isolation, properties and karyotype analysis of pluripotent (EK) cell lines from normal and parthenogenetic embryos. In: Silver LM, Martin GR, Strickland S (eds.), Teratocarcinoma Stem Cells. Cold Spring Harbor Conference on Cell Proliferation. New York: Cold Spring Harbor Laboratory; 1983: 10:647–663.
27. Eistetter HR. Pluripotent embryonal stem cells lines can be established from disaggregated mouse morulae. Dev Growth Differ 1989; 31:275–282.
28. Sukoyan MA, Vatolin SY, Golubitsa AN, Zehelezova AI, Semenova LA, Serov OL. Embryonic stem cells derived from morulae, inner cell mass, and blastocyst of mink: comparisons of their pluripotencies. Mol Reprod Dev 1993; 36:148–158.
29. Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G, McDermand D. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. Theriogenology 1991; 35:161–170.
30. Stice SL, Keefer CL. Multiple generational bovine embryo cloning. Biol Reprod 1993; 48:715–719.